Cytotoxic Activity Against Small Cell Lung Cancer Cell Line and Chromatographic Fingerprinting of Six Isolated Compounds from the Ethanolic Extract of Benjakul

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Background: *Benjakul, a Thai traditional herbal preparation, comprises five plants: Piper chaba, Piper sarmentosum, Piper interruptum, Plumbago indica, and Zingiber officinale. It has widely been used to treat cancer patients in folk medicine in Thailand. Benjakul extract, and its isolated compounds should be investigated for cytotoxic activity and analysis isolated compounds from chemical fingerprinting.*

Objective: *To study cytotoxicity of Benjakul extract and its isolated pure compounds against human small cell lung cancer cell line (NCI-H1688) and in normal human lung fibroblast cell line (MRC-5) and analysis the content of isolated compounds for quality control of Benjakul extract.*

Material and Method: *Bioassay-guided fractionation was used for isolated active compounds from ethanolic extract of Benjakul. Cytotoxic activity was carried using the SRB assay. HPLC method was applied to analyze six isolated compound content from Benjakul extract.*

Results: *The ethanolic extract of Benjakul showed cytotoxicity against NCI-H1688 with IC_{50} value = 36.15±4.35 μg/ml. Hexane fraction as semi-separation by VLC showed the best cytotoxic activity (21.17±7.42 μg/ml). Six isolated compounds were identified as myristicin, plumbagin, methyl piperate, 6-shogaol, 6-gingerol and piperine. Plumbagin exhibited the highest cytotoxic activity and 6-shogaol was the second most effective cytotoxic constituent (IC_{50} values = 1.41±0.01 and 6.45±0.19 μg/ml, respectively). Piperine showed the highest content in both of HPLC analysis and column chromatography separation.*

Conclusion: *Benjakul extract exhibited cytotoxicity against NCI-H1688. Plumbagin and 6-shogaol are bioactive markers for cytotoxicity against this small cell lung cancer cell line. Chromatographic fingerprinting can be used to analyze six cytotoxic compounds isolated from the ethanolic extract of Benjakul.*

Keywords: *Benjakul, Cytotoxic activity, NCI-H1688, Chromatographic fingerprint*

J Med Assoc Thai 2014; 97 (Suppl. 8): S70-S75
Full text. e-Journal: *http://www.jmatonline.com*

Benjakul preparation is a known herbal remedy used for balance the four basic elements in Thai traditional medicine and treatment cancers in folk medicine. This preparation is also used as an adaptogen and for treatment of dyspepsia in the lists of the National Drug List of Herbal Medicine Products AD[1]. Benjakul preparation comprised five plants: fruit of *Piper chaba*, root of *Piper samentosum*, stem of *Piper interuptum*, root of *Plumbago indica* and rhizome of *Zingiber officinale*, in equal proportions. The previous report of the ethanolic extract of Benjakul found cytotoxicity against lung cancer cell, COR-L23[2]. Piperine, plumbagin and 6-gingerol were isolated from the ethanolic extract of Benjakul[3]. Furthermore, piperine and 6-gingerol showed highest cytotoxicity against breast cancer cell (MCF-7)[4] and HPLC method for

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quantitative determination of piperine and plumbagin have been reported[5].

In the present study, piperine, plumbagin, 6-gingerol and the other three components were isolated from the ethanolic extract of Benjakul. Cytotoxicity of six isolated compounds against human small cell lung cancer cell line (NCI-H1688) was determined by using SRB assay. In addition, all of those compounds were investigated for quality control of Benjakul extract by HPLC.

Material and Method

Reagents and materials

Authentic compounds: plumbagin was purchased from Sigma-Aldrich (St. Louis, Missouri, USA); 6-gingerol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); piperine was purchased from Merck (Darmstadt, Germany). Silica gel 60 (0.063-0.200 mm and 0.040-0.060 mm), hexane, chloroform and methanol were purchased from Merck (Darmstadt, Germany). Acetronitrile and purified water (HPLC grade) were purchased from RCI Labscan (Bangkok, Thailand). Trypsin-EDTA, fetal bovine serum (FBS), the antibiotics penicillin and streptomycin, Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium and phosphate buffered saline (PBS) were purchased from Gibco®, Life Technologies (New York, USA). DMSO, Tris base and SRB were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Plant materials and preparation of extract

Five plants of Benjakul preparation: fruits of *Piper chaba* Hunt (Piperaceae), root of *Piper sarmentosum* Roxb. (Piperaceae), stem *of Piper interruptum* Opiz. (Piperaceae), root of *Plumbago indica* Linn. (Plumbaginaceae) and rhizome of *Zingiber officinale* Roscoe. (Zingiberaceae) were collected in Chantaburi Province in August, 2010 and authenticated by comparison with herbarium specimens at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand.

All plants were cleaned immediately of extraneous material and dried at 50°C. A sample (1 kg) of the each dried plant material was coarsely powdered then pooled together and macerated at room temperature with 95% ethanol (30 Lx3, 3 days each). The extracts were pooled and concentrated by using a rotary evaporator (Rotavapor R-205, Buchi, Switzerland) and then freeze-dried (Lyolab LT, Lyophilization systems, Inc. USA). The percentage of yields was 9.11.

Isolation and structure elucidation of compounds from the ethanolic extract of Benjakul

The ethanolic extract of Benjakul (60 g) was separated to be five fractions using vacuum liquid chromatography (VLC) on silica gel 60 (Merck, 70-230 mesh) by ordering increase polarity of solvents: hexane 2,000 ml, hexane: chloroform (1:1) 2,000 ml, chloroform 2,500 ml, chloroform: methanol (1:1) 2,000 ml and methanol 2,000 ml. Drying and evaporation of each fraction yielded residues of 1.23 g, 1.33 g, 12.44 g, 31.95 g and 10.01 g, respectively. Cytotoxicity of five fractions against small lung cancer cell line (NCI-H1688) was determined by using the SRB assay and chloroform fraction, which showed the high cytotoxic activity and high percentage w/w yield was further purified using column chromatography.

The dried chloroform fraction (5 g) from VLC was subjected to silica gel column (Merck, 230-400 mesh), elution was carried out using a mixture of hexane: ethyl acetate gradient system (9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8, 1:9), followed with ethyl acetate: methanol gradient system (8:2, 7:3, 6:4) to yield 271 fractions, following TLC examination and detection with acidic anisaldehyde spray, fractions containing the same constituents were combined. Then they were further purified by column chromatography combined with thin layer chromatography and six pure compounds were separated. Compound 1, 3, 4 were identified by their NMR data [^{1}H and ^{13}C on a Varian Unity Inova 500 spectrometer (500 MHz for ^{1}H; 125 MHz for ^{13}C)], EI mass spectra, Low resolution were obtained from a JEOL JMS-AX505W spectrometer. Compound 1 was identified as myristicin[6], compound 3 was methyl piperate[7] and compound 4 was 6-shogaol[8] by comparison with data in these three reports. Compounds 2, 5 and 6 gave identical spectra by HPLC to authentic samples of plumbagin (Sigma-Aldrich), 6-gingerol (Wako) and piperine (Merck), respectively.

HPLC fingerprint and determination of compounds in the ethanolic extract of Benjakul

The analysis of the ethanolic extract of Benjakul was carried out using high performance liquid chromatography, HPLC (Constametric® 4100 Bio) with ultraviolet visible detector (Spectromonitor® 4100) and automatic injector (Spectra System AS3500). A reversed-phase column was Phenomenex Luna 5 µm C18(2) 100A analytical column (250x4.6 mm 5 micron; Phenomenex, Inc., USA), protected by a Security Guard Cartridge (C18, 4x3 mm; Phenomenex, Inc., USA). The condition was followed by Itharat and

Sakpakdeejaroen[5]. In briefly, the mobile phase was water-acetonitrile with gradient elution as follows: 0 min, 60:40; 30 min, 50:50; 50 min, 5:95; 60 min, 0:100. Elution was carried out at a flow rate of 1.0 ml/min and detected with a wavelength for UV detection at 256 nm. The operating temperature was maintained at room temperature.

Benjakul extract was dissolved in methanol, sonicated and filtered through a 0.45 µm membrane filter before use. The content of the active compounds from Benjakul extract were determined using a calibration curve established with five dilutions of each standard. All samples were analyzed in triplicate. The corresponding peak areas were plotted against the concentration of the compound injected. Peak identification was achieved by comparison the retention time (RT) with those obtained for standards. The quantification was achieved by comparison with the calibration curves.

Cell culture

The human small cell lung cancer cell line (NCI-H1688) and normal human lung fibroblasts cell line (MRC-5), kindly provided by Asst. Prof. Dr. Pintusorn Hansakul, Faculty of Medicine, Thammasat University, Thailand, were cultured in RPMI 1640 medium (GIBCO™) and Dulbecco's modified Eagle's (DMEM), respectively. They were supplemented with 10% fetal bovine serum (GIBCO™), 50 IU/ml penicillin and 50 µg/ml streptomycin (GIBCO™)[9,10] in a 95% humidity incubator at 37°C with 5% CO_2.

Cytotoxicity testing

Growth inhibition of NCI-H1688 and MRC-5 cells were determined by using the modified SRB assay as described by Skehan et al[11]. Briefly, cells were seeded at a density of $1x10^3$ and $5x10^3$ cells/well for NCI-H1688 and MRC-5, respectively, in 96-well plates. After 24 h, serial dilutions of samples (Benjakul extract, VLC fractions and pure compounds) and standard drug (Paclitaxel®) in DMSO were added for each concentration. 2% of DMSO being used in solvent control wells. The cells were exposed to test samples for continuous 72 h. After that, the medium was removed, the wells were washed with medium, and 200 µl of fresh medium were then added. The plates were incubated at 37°C for a recovery period of 72 h. For cell fixation, the cold trichloroacetic (40%, 100 µl) was added in each plate and incubated at 4°C for 1 h. Then the plates were washed with cold water. SRB stain (50 µl; 0.4 in 1% acetic acid) was added for 30 min and unbound dye was washed out with 1% acetic acid 5 times. After air-drying at room temperature, SRB dye within cells was dissolved with 100 µl of 10 mM Tris base (pH 10.5). The optical density of the extracted SRB dye was measured with a power wave X plate reader at 492 nm. The 50% inhibitory concentration (IC_{50}) was calculated from the Prism program obtained by plotting the percentage of survival versus the concentrations. All individual experiments were carried out in quadruplet for each concentration. All data are presented as means $\pm$ SEM of three separate experiments carried out on different days.

Results and Discussion

The percentage of yields and cytotoxic activity of Benjakul extract, each fraction from VLC and pure compounds are showed in Table 1.

The ethanolic extract of Benjakul showed cytotoxicity against NCI-H1688 than MRC-5 with IC_{50} values being 36.15 ± 4.35 and 70.07 ± 2.46 µg/ml, respectively. These data are consistent with a previous finding that Benjakul extract show cytotoxicity against COR-L23 (IC_{50} = 19.80 µg/ml)[2]. This result is the first report that study in small cell lung cancer. This report can conclude that Benjakul extract showed more selective cytotoxicity against COR-L23 (non-small cell lung cancer) than NCI-H1688. Furthermore, Benjakul extract can kill cancer cells more than normal cells (selective index = 1.94), whereas paclitaxel killed more normal cells than cancer cells (selective index = 0.20). In addition, hexane and chloroform fraction from VLC showed higher potency against NCI-H1688 than Benjakul extract. It is indicated that semi-purification by hexane extract showed higher selective NCI-H1688. Interestingly, this fraction showed the highest selective index as 3.44. The characteristic of hexane fraction was oil, so direct Benjakul maceration with hexane should be continuously studied for cytotoxicity against small cell lung cancer cell line and isolate active cytotoxic compounds. The chloroform fraction, which showed high cytotoxic activity and high yields, and also selective index more than 1, was selected to isolate pure compounds by column chromatography. The chemical structures of six pure compounds were identified as follows: compound 1 (3.5 mg) as myristicin, 2 (32.4 mg) as plumbagin, 3 (25.5 mg) as methyl piperate, 4 (8.8 mg) as 6-shogaol, 5 (9.6 mg) as 6-gingerol and 6 (273.8 mg) as piperine. The yield of six compounds which isolated from column chromatography were calculated as mg/g of extraction, they are showed in Table 2.

Plumbagin and 6-shogaol showed the most and the second most cytotoxicity against NCI-H1688 (IC_{50} = 1.41±0.01 and 6.45±0.19 µg/ml, respectively). These data are also related with previous study on cytotoxicity of plumbagin and 6-shogaol against A549 with IC_{50} = 2.75 and 1.47 µg/ml (14.6 and 5.32 µM), respectively[8,12]. These results are also reported as the first time for six compounds against NCI-H1688.

Table 1. Percentage yields and cytotoxic activity of ethanolic extract, each fractions from VLC and isolated compounds from Benjakul preparation against small lung cancer cell line (NCI-H1688), and normal human lung fibroblast cell line (MRC-5)

Samples	Yield (%w/w of extract)	IC_{50} (µg/ml)[a]		
		NCI-H1688	Selective index[b]	MRC-5
Ethanolic extract of Benjakul preparation		36.15±4.35	1.94	70.07±2.46
Hexane fraction	2.04	21.17±7.42	3.44	72.49±5.98
Hexane: chloroform fraction	2.24	52.10±1.04	0.71	37.27±8.98
Chloroform fraction	20.73	23.42±0.32	1.34	31.44±1.98
Chloroform: methanol fraction	53.25	40.24±8.76	1.86	74.79±8.39
Methanol fraction	16.71	>100	-	>100
Myristicin (1)	0.01	>100 520.26 µM	<0.80	80.69±5.40 419.80 µM
Plumbagin (2)	0.13	1.41±0.01 7.49 µM	0.68	0.97±0.58 5.15 µM
Methyl piperate (3)	0.10	>100 >430.61 µM	<0.58	57.99±10.38 249.69 µM
6-Shogaol (4)	0.04	6.45±0.19 23.34 µM	0.79	5.12±0.50 18.53 µM
6-Gingerol (5)	0.04	51.24±10.49 174.05 µM	1.74	89.33±10.50 303.44 µM
Piperine (6)	1.12	32.73±0.43 114.71 µM	1.89	38.83±4.97 136.08 µM
Paclitaxel[c]		0.058±0.07 50.068 µM	0.20	0.012±0.01 10.014 µM

[a]IC_{50} values are expressed as the mean ± SEM of 4 replicates from three separate experiments; [b]selective index = IC_{50} of normal cell (MRC-5)/IC_{50} of cancer cell (NCI-H1688); [c]positive control

Table 2. The concentration range, linearity (r^2), retention time (RT), and content (mg/g of extract) of bioactive marker isolated from Benjakul extract which analyzed by using high pressure liquid chromatography (HPLC) and column chromatography (CC)

Peak number[a]	Compounds	Concentration range (µg/mL)	Linearity (r^2)	RT (min)		Content (mg/g of extract)	
				standard	Benjakul extract	HPLC[b]	CC
1	6-Gingerol	50-400	0.9995	19.035	18.897	11.27±0.76	0.39
2	Plumbagin	25-200	0.9999	20.802	20.701	5.55±0.40	1.33
3	Piperine	50-400	0.9999	23.488	23.341	97.46±6.06	11.12
4	Methyl piperate	50-400	0.9994	29.921	29.734	3.07±0.15	1.04
5	Myristicin	50-400	0.9992	33.134	33.004	6.83±0.52	0.39
6	6-Shogaol	50-400	0.9992	40.620	40.541	12.00±0.46	0.36

[a]Peak number refers to peak chromatogram in Fig. 1; [b]Mean values ± SD (n = 3)

Although both of plumbagin and 6-shogaol have nonspecific cytotoxic activity (selective index less than 1), but their selective indices were of higher value than paclitaxel. This suggested that they killed less normal cells than paclitaxel. Thus, plumbagin and 6-shogaol should be bioactive markers for the ethanolic extract of Benjakul.

Chromatograms of Benjakul extract and standard compounds are showed in Fig. 1. The results from HPLC compared with column chromatography are showed in Table 2. The content of six compounds was calculated from chromatographic fingerprint and standard curve (linearity or r^2 showed in Table 2). Retention time of standard compounds and Benjakul extract were applied as internal and external standard technique. The content of isolated compounds, which were isolated from column chromatography was compared with content from HPLC. These data suggested that method for isolation pure components by column chromatography obtained less than 9-33 times. Content of cytotoxic compounds as plumbagin and 6-shogaol were 5.5 and 12 mg/g of extract calculated by HPLC. Piperine is the main compound and showed the highest content as 97.46 mg/g of extract. From the previously report, piperine and plumbagin showed less content than this research (46.71 and 2.46 mg/g of extract, respectively)[5]. This chromatographic fingerprinting of Benjakul extract with six isolated compounds was reported for the first time.

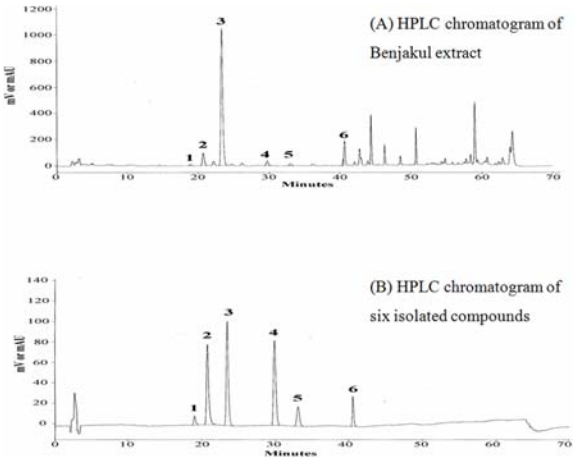

Fig. 1 (A) HPLC chromatogram of ethanolic extract of Benjakul (10 mg/ml) compared with (B) HPLC chromatogram of six isolated compounds that separated from ethanolic extract of Benjakul. Peak from chromatograms were described as follow: peak number 1 = 6-gingerol, 2 = plumbagin, 3 = piperine, 4 = methyl piperate, 5 = myristicin, 6 = 6-shogaol.

Conclusion

The results indicate that Benjakul preparation contains a number of active compounds that may be responsible for cancer therapeutic activity. The HPLC method developed will assist in the standardization of Benjakul preparation using bioactive markers.

Acknowledgement

The authors wish to thank the National Research University Project of Thailand Office of Higher Education Commission and Center of Excellence on Applied Thai Traditional Medicine Research (CEATMR), Thammasat University, Thailand, for financial support.

Potential conflicts of interest

None.

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ฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอดชนิด small cell lung cancer และลายพิมพ์นิ้วมือของสารสำคัญ 6 ชนิด ที่แยกได้จากสารสกัดตำรับเบญจกูล

รุจิลักขณ์ รัตตะรมย์, อินทัช ศักดิ์ภักดีเจริญ, พินทุสร หาญสกุล, อรุณพร อิฐรัตน์

ภูมิหลัง: *ตำรับเบญจกูลประกอบด้วยสมุนไพร 5 ชนิด ได้แก่ ดีปลี ช้าพลู สะค้าน เจตมูลเพลิงแดง และขิง เป็นตำรับยาที่ใช้เป็นส่วนประกอบในการรักษาโรคมะเร็งโดยหมอพื้นบ้านในภาคใต้ของไทย สารสกัดตำรับเบญจกูลและสารสำคัญที่แยกได้จากตำรับ นำไปศึกษาฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งและหาปริมาณสารสำคัญในตำรับโดยใช้ลายพิมพ์นิ้วมือ*

วัตถุประสงค์: *เพื่อศึกษาฤทธิ์ความเป็นพิษต่อเซลล์ของสารสกัดตำรับเบญจกูลและสารสำคัญที่แยกได้จากตำรับในมะเร็งปอดชนิด NCI-H1688 เปรียบเทียบกับเซลล์ปอดปกติและพัฒนาวิธีการสร้างลายพิมพ์นิ้วมือของตำรับเบญจกูล และวิเคราะห์ปริมาณของสารสำคัญที่แยกได้จากตำรับ*

วัสดุและวิธีการ: *แยกสารสำคัญที่มีฤทธิ์จากสารสกัดตำรับเบญจกูลด้วยวิธี bioassay-guided fractionation โดยเทคนิคโครมาโตกราฟี ศึกษาฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอดด้วยวิธี SRB และวิเคราะห์ปริมาณของสารสำคัญโดยใช้เทคนิค HPLC*

ผลการศึกษา: *สารสกัดตำรับเบญจกูลและสารสกัดย่อยส่วน hexane มีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอด NCI-H1688 (IC_{50} = 36.15±4.35 และ 21.17±7.42 ไมโครกรัม/มิลลิลิตรตามลำดับ) สารสำคัญที่แยกได้จากตำรับเบญจกูล 6 ชนิด ได้แก่ myristicin, plumbagin, methyl piperate, 6-shogaol, 6-gingerol และ piperine พบว่า สาร plumbagin มีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอด NCI-H1688 มากที่สุด รองลงมาคือสาร 6-shogaol สาร piperine มีปริมาณสูงที่สุดในสารสกัดตำรับเบญจกูล เมื่อวิเคราะห์ด้วยเทคนิค HPLC และ column chromatography*

สรุป: *สารสกัดเบญจกูลมีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอด NCI-H1688 สาร plumbagin และ 6-shogaol ซึ่งมีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอด NCI-H1688 สามารถใช้เป็น bioactive markers ในการวิเคราะห์ปริมาณสารสำคัญในตำรับเบญจกูลได้โดยใช้ลายพิมพ์นิ้วมือของสารสกัดเบญจกูลในการวิเคราะห์ปริมาณสารสำคัญที่แยกได้*